Institute of Experimental Morphology, Pathology and Anthropology with Museum
Bulgarian Anatomical Society

Acta morphologica et anthropologica, 25 (3-4)
Sofia ● 2018

Morphology of Mesenchymal Stem Cells in 3D spheroids

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The conventional method of cell cultivation in plasticware leads to the formation of a monolayer of cells with changed morphology and intercellular communications. Here, the method of the hanging drop is used as a technique for 3D cultivation of adipose tissue-derived mesenchymal stem cells to study the morphological changes of cells, which compose the spheroids. Three cell lines were investigated and were shown to form spheroids in hanging drops after 24 hours. A time-dependent decrease of x and y dimensions of the spheroids and a simultaneous increase of z dimension were observed. Changes in morphology and the nuclei volume of the cells at day 3 and day 5 in 3D culture compared to monolayer cell culture were observed indicating the importance of the type of culture conditions (2D and 3D) for the communications and physiology of the cells.

Key words: Mesenchymal stem cells, 3D, Spheroids, Spheroid size, Nuclei size

Introduction

Mesenchymal stem cells (MSC) are multipotent stem cells which exist in different tissues of adult organisms. They are self-renewable, and can easily be isolated and expanded for long periods *in vitro*, without losing their major biological characteristics. Initially, MSC have been isolated from bone marrow, but later on techniques have been elaborated for isolation of MSCs from almost all other tissues. To date, there are no MSC-specific markers known; therefore, the International Society for Cellular Therapy has defined three criteria, which should be fulfilled in order to assign a cells to MSC type. These criteria are: (a) ability of adherence to lab plasticware; (b) expression of specific surface antigens ($CD73^+$, $CD90^+$ and $CD105^+$) and lack of expression of hematopoietic antigens, such as ($CD45^-$, $CD34^-$, $CD11b^-$ and HLA class II); and (c) potential for differentiation in different cell types, such as osteoblasts, adipocytes and chondrocytes [5]. Due to their multipotency, MSC are a promising candidate for vast applications in regenerative medicine. Moreover, this type of stem cells has the ability to migrate to the sites of injured tissues in the organism where they help tissue recovery by secreting various growth factors and cytokines. Other significant biological property of MSC is their participation as immunomodulators in the places of inflammation [summarized in 8].

In vitro, MSC are usually maintained in long-term 2D condition without undergoing any significant abnormalities. MSC adhere to plastic labware, proliferate and form monolayer during cultivation. This artificial condition of maintenance of MSC is different from the natural microenvironment in the organism and leads to atypical morphology of some cell types, when they are cultured in a monolayer. Furthermore, this way of cell culturing limits cell-to-cell contacts and the opportunities of the cells to form natural structures specific for organisms. The three-dimensional (3D) cell culture creates an environment in which the cells grow and interact among them in the space [4]. There are different techniques for 3D culturing but the hanging drop method is the easiest and the most commonly used. A cell suspension with a defined concentration is placed on the lid of a Petri dish. This prevents the cells from adherence to the lab plasticware and leads to the formation of three-dimensional structure called spheroid. These 3D cell structures provide closer to the natural environment for the cell maintenance compared to the 2D monolayer culturing, and are a better model for studies in regenerative medicine, cell therapies, drug testing, tumor biology and stem cell research [3].

The purpose of this work is to investigate the process of formation of 3D cell structures (spheroids) by adipose tissue-derived mesenchymal stem cells (ASC) as well as to study the occurring morphological changes of the cells when they are cultured in 3D condition.

Material and Methods

Cell Cultures. Human mesenchymal stem cells from adipose tissue (ASC) have been isolated, characterized and cultured in DMEM supplemented with 10% fetal calf serum and 1% Penicillin, Streptomycin, Amphotericin B (Sigma) in 2D culture condition to achieve a confluent monolayer. The method of the hanging drop was used for spheroid formation. Thirty-five µl cell suspension drops (25 000 cells/drop) were placed and cultured on the lid of Petri dishes at 37°C, 5%CO_2.

Light microscopy. The spheroids were taken on pictures every 24 hours from day 1 to day 6 of cultivation via LeicaDMI 3000B. The spheroids' dimensions (x; y) have been measured [µm] by LAS version 3.4.0 software. The data was analysed by Excel.

Confocal microscopy. Spheroids in hanging drops have been prepared for confocal microscopy on day 3 and day 5. Nuclei staining was performed by 20 µg/ml Hoechst 33258 (Sigma) in 1xPBS for 1h at room temperature. The spheroids were washed three times in 1xPBS and mounted on slides with Eukitt® quick hardening mounting medium (Sigma). 3D structures have been scanned: 1) whole spheroids and 2) the periphery and the center area of the spheroids. The size (x; y; z) of the nucleus of each cell was measured by ImageJ in different parts of the spheroids. The data was analyzed in Excel.

Statistical analysis. Standard data deviations were calculated in Excel and are shown on figures.

Results

Spheroids in hanging drops in 35 µl culture medium and concentration 25 000 cells/drop were prepared from three primary cell cultures of adipose tissue-derived mesenchymal stem cells (ASC07, ASC08 and ASC10). The formation of spheroid-like structures from each cell line was detected after 24 hours via light microscopy. One spheroid was formed in each

drop and its morphology and structure were observed till day 6 (**Fig. 1A**). All spheroids were organized in compact, round structure with smooth edges. The size of the spheroids (x; y) was measured every 24 hours from day 1 to day 6 (**Fig. 1B, C and D**). All spheroids decreased in size (x; y) during the 6 day period of culturing showing changes in the range from 450-500 µm/diameter at day 1 to 290-350 µm at day 6. The change of the size was different for the spheroids formed by different cell lines. ASC07 spheroids decreased the most showing a reduction of 30%: from ~450µm/diameter at day 2 to ~290µm/diameter at day 6, while the spheroids from the other two ASC primary cultures shrank to lesser extent.

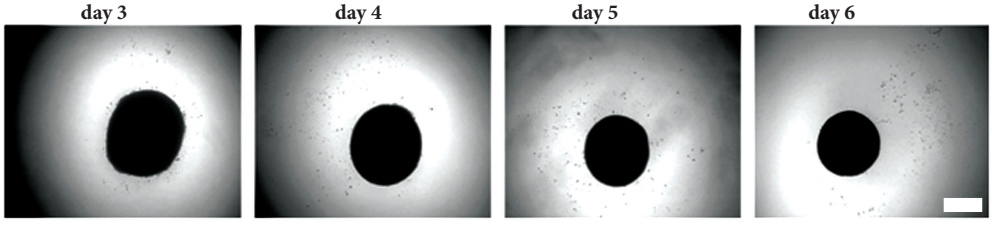


Fig. 1A

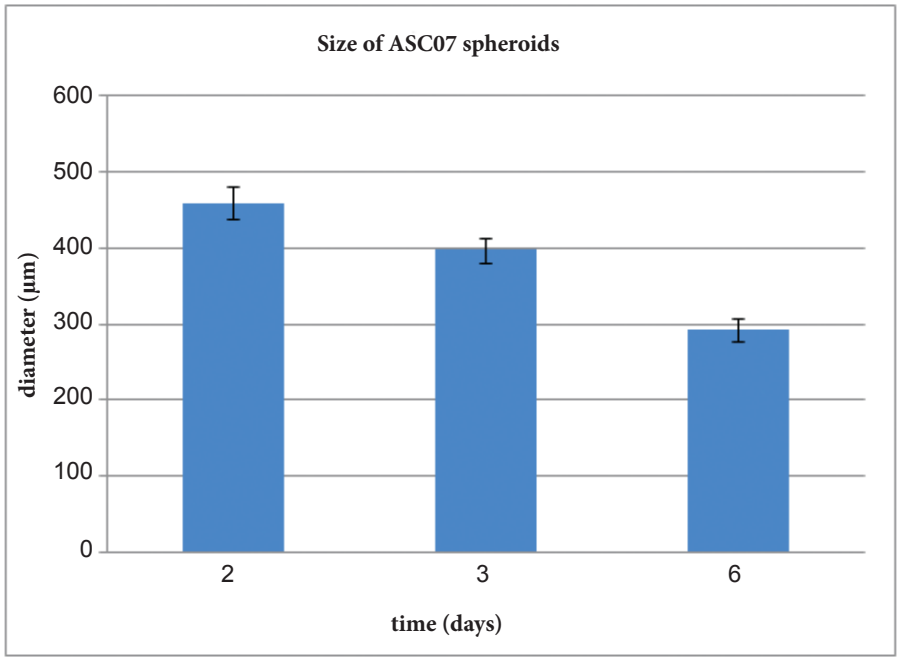


Fig. 1B

Fig. 1C

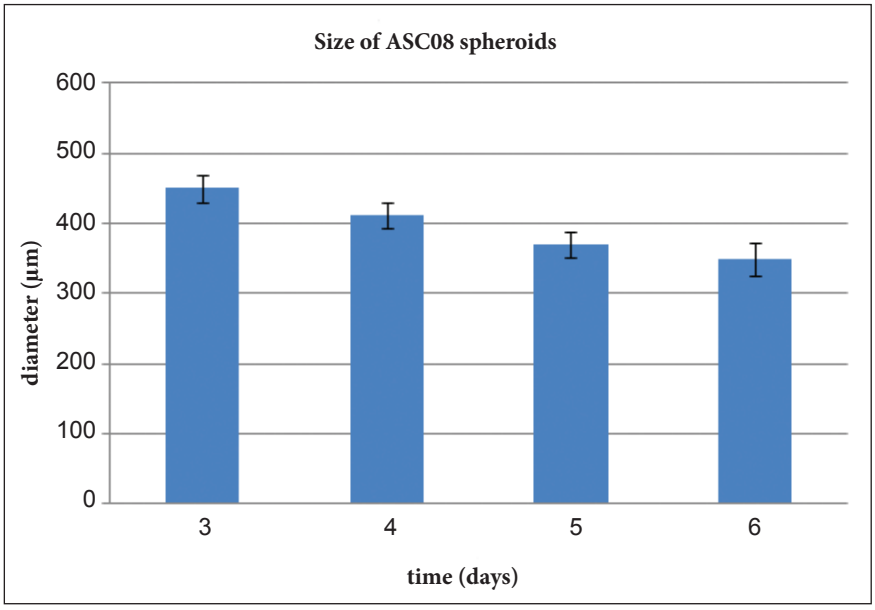


Fig. 1D

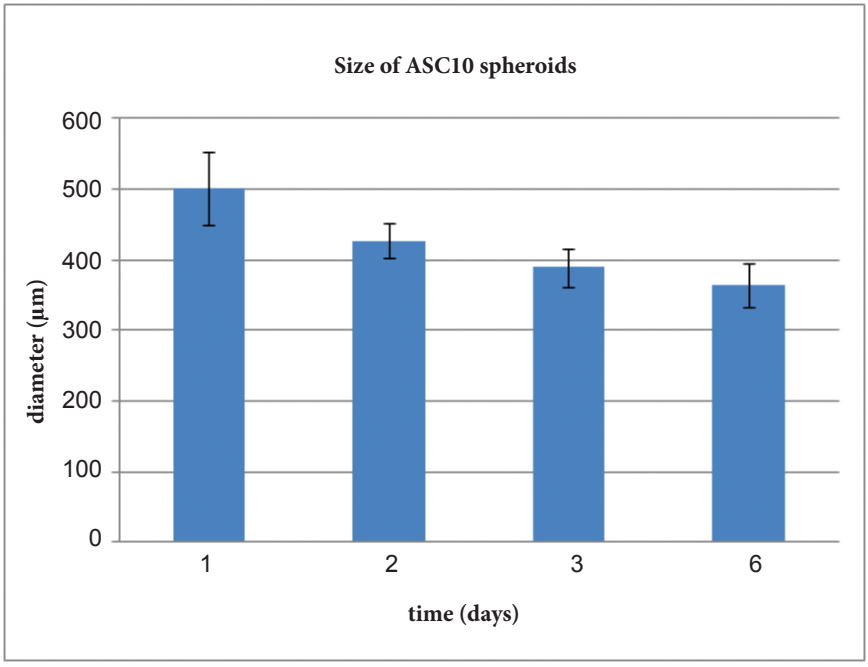
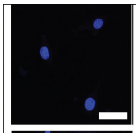
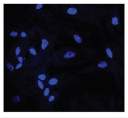


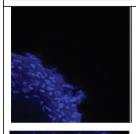
Fig. 1. Morphology of ASC spheroids. (A) Light microscopy of ASC08 spheroids from day 3 to day 6; Charts of average diameter (µm) of ASC07 spheroids, n = 20 (B); ASC08 spheroids, n = 58, at day 2 and day 3; n = 18, at day 5 and day 6 (C); and ASC10 spheroids, n = 22 (D) measured every 24 hours from day 1 to day 6. Scale bar = 200 µm



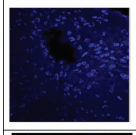
2D single cells	Nuclei diameter (µm)	Cell area diameter (µm)
Mean	12.974	38.151
SD	3.316	20.433
Min	7.488	11.264
Max	22.645	86.72



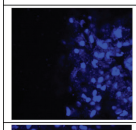
2D monolayer	Nuclei diameter (µm)	Cell area diameter (µm)
Mean	12.09	36.731
SD	2.618	9.676
Min	6.671	16.682
Max	18.333	64.262



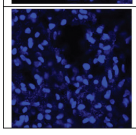
3D day 3 - periphery	Nuclei diameter (µm)	Cell area diameter (µm)
Mean	5.841	8.905
SD	1.232	1.74
Min	2.641	5.213
Max	9.712	14.296



3D day 3 - core	Nuclei diameter (µm)	Cell area diameter (µm)
Mean	6.25	8.096
SD	1.337	1.394
Min	3.597	4.195
Max	10.792	10.937



3D day 5 - periphery	Nuclei diameter (µm)	Cell area diameter (µm)
Mean	7.146	9.403
SD	1.299	1.69
Min	4.438	6.014
Max	10.671	15.541



3D day 5 - core	Nuclei diameter (µm)	Cell area diameter (µm)
Mean	7.959	11.413
SD	1.164	1.921
Min	5.276	6.479
Max	11.151	16.069

Fig.2. Comparison between dimensions of ASC10 cell nuclei in 2D and 3D culture conditions. Left panel: confocal microscopy of cells in 2D conditions compared to cells in 3D spheroids (nuclei staining – Hoechst 33258). Right panel: Table of average diameter (µm) of nuclei and cell area in 2D and 3D conditions. Scale bar = 50 µm

Furthermore, a comparison of cell morphology between 2D and 3D culture conditions was performed. The dimensions of nuclei and cytoplasm (**Fig. 2**) were studied. First, ASC10 cell line was used to compare the size of nuclei and cytoplasm of non-confluent to confluent ASC grown in 2D conditions. The results showed no significant difference of nuclei and cytoplasm in both culture conditions. In average the size of nuclei was ~12-13 µm/diameter and cell area ~36-38µm in diameter independent of the confluence of ASC. Nevertheless, a distinctive difference in the morphology of ASC nuclei was observed. In the non-confluent cultures the nuclei had more rounded shape unlike the elongated nuclei of ASC in confluent state of culture. Moreover, we measured the size of ASC nuclei in the 3D spheroids and compared the results with 2D culture. At day 3 and day 5 the size of ASC nuclei in the periphery and the center of spheroids were compared. The results showed a significant decrease of the nuclei dimensions (x; y), which changed from ~12-13 µm in 2D to ~6-8µm in 3D. Also taking into account the z dimention, the volumes of nuclei showed even more significant decrease: from 310-360 μm^3 in 2D to 134 μm^3 in the center and 109 μm^3 in the periphery of 3D spheroids on day 3. On day 5, the nuclei doubled their volumes in 3D cultures becoming 273 μm^3 in the center and 197 μm^3 in the periphery of the spheroids. Similar results were also observed for the cells size (**Fig. 2**).

The morphology of the spheroids is presented by confocal microscopy on **Fig. 3**. While the light microscopy revealed decrease in x and y dimensions of 3D spheroids during the culturing, the measurement of z dimention by confocal microscopy showed an increase of the spheroids from 40-50 µm at day 2 and day 3 to 150 µm at day 6. In addition the confocal imaging revealed the formation of cavities in the center of some spheroids.

Discussion

Hanging drop method for spheroid formation eliminates surface attachment by placing the cell suspension in a drop, allowing gravity to facilitate cellular aggregation at the bottom of the drop [6]. Here, our results showed that ASC aggregated at the bottom of the drop in the first day of spheroids formation. Confocal imaging revealed a sheet-like aggregation of ASC in the spheroids when investigated in z dimention at day 3. At day 6 the structures significantly increased in z axis. Corresponding to this result, the nuclear

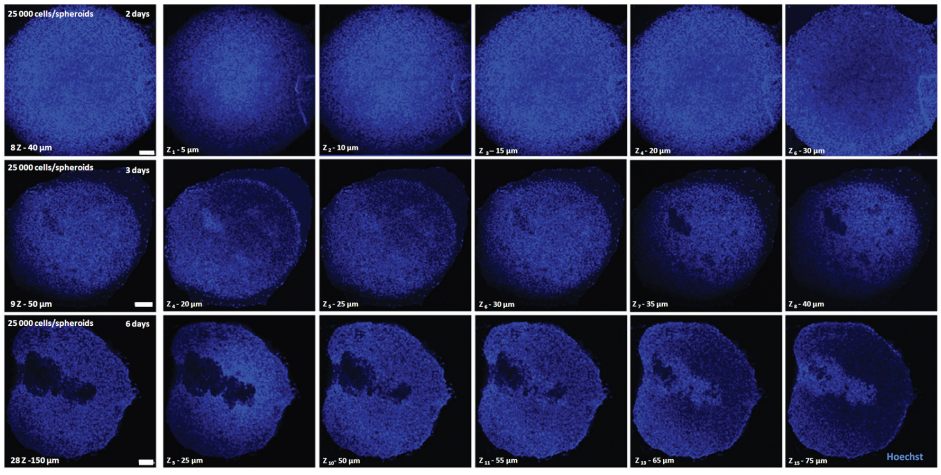


Fig. 3. Confocal microscopy of ASC07 spheroids (nuclei staining – Hoechst 33258): Changes of structure and thickness (on z axis) of spheroids on day 2, day 3 and day 6. Scale bar = 100 µm

volumes initially decreased by 72% till day 3, and thereafter doubled their volume by day 5, when compared to 2D culturing. A significant 75% reduction of the size of MSCs in spheroids compared to cells in 2D monolayer was reported by others [1, 2, 10, 11]. Our observation that nuclei volume reduces to the same extend confirms the data from the literature. We have not observed more elongated cells in the periphery of the spheroids, reported by Zhang Q [11], but the nuclei dimensions in the center and in the periphery of the spheroids are slightly different. The nuclei of the cells in the center are larger than those in the edges of the spheroids. Additionally, the shape of the nuclei changed and the cells in the spheroids showed sphere-like shape of nuclei, while the cells in the monolayer had ellipsoid-like nuclei. In MSC biology cellular morphology is a key characteristic used to determine cellular phenotypes, ability for differentiation and fates of MSC. Cytoskeletal reorganization and drastic changes in cell morphology in MSC spheroids indicate a major difference in mechanophysical properties compared with 2D culture [3].

Now it is well known that conventional methods of cell cultivation provide conditions which are very different from the natural environment of the cells. The necessity of adding a third dimention to the cell culture system, more closely resembles the natural environment and might generate significant differences in MSCs phenotype, behavior and therapeutic potential, which is increasingly recognized in the literature [7, 9].

Conclusion

The main observations in this work are the reduction of x and y dimensions of ASC spheroids during the culturing and simultaneous enlargement in z direction; nuclei volumes in 3D on day 3 lessening to 38%, compared to 2D, but doubled between day 3 and 5.

Acknowledgement: This research was supported by grant DCOST01/18, NSF, Bulgaria.

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